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Using Ca²⁺-channel biosensors to profile amphetamines and cathinones at monoamine transporters: electro-engineering cells to detect potential new psychoactive substances.

Tyler W. E. Steele and Jose M. Eltit*

Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University.

Abstract

Background—The appearance of stimulant-class new psychoactive substances (NPS) is a frequent and significant problem in our society. Cathinone variants are often sold illegally as 3,4-methylenedioxymethamphetamine (“ecstasy”) or disguised for legal sale using misleading names such as “bath salts” and carry the risk of promoting disruptive mental states, addiction, and fatal overdose. The principal targets of these recreational drugs are monoamine transporters expressed in catecholaminergic and serotonergic neurons. Some transporter ligands can be transported into cells, where they can promote a massive release of neurotransmitters through reverse transport, and others can block uptake. A ligand’s dopamine vs. serotonin transporter selectivity, potency, and activity as a substrate or blocker can help elucidate the abuse liability and subjective effects of a drug.

Objectives—Here we describe the discovery, development, and validation of an emerging methodology for compound activity assessment at monoamine transporters.

Key findings—Substrates generate inward electrical currents through transporters, and can depolarize the plasma membrane, whereas blockers work as a “cork in a bottle” and function as antagonists. Voltage-gated Ca²⁺ channels were co-expressed with monoamine transporters in cultured cells and used to measure fluctuations of the membrane electrical potential. In this system, substrates of monoamine transporters produce reliable dose-dependent Ca²⁺ signals while blockers hinder them.

Discussion—This system constitutes a novel use of voltage-gated Ca²⁺ channels as biosensors for the purpose of characterizing ligand activity at monoamine transporters using fluorimetry. This approach in combination with *in vivo* evaluations of drugs’ abuse-related effects is a powerful strategy for anticipating potential stimulant-class NPS.

Keywords

DAT; NET; SERT; psychostimulants; screening; MDPV; drugs of abuse; cocaine; methcathinone; calcium channels

*Corresponding author: 1101 E Marshall St. Rm# 3-038H, Richmond VA, 23298, Phone: (804)628-4844, Fax: (804)828-7382, jose.eltit@vcuhealth.org.

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Brief overview of amphetamines and cathinones

Naturally occurring secondary metabolites found in various plant and fungus species target the human central nervous system when consumed and alter its function. For centuries, consumption of these natural products was limited to raw extracts, but the rise of modern chemistry has generated a myriad of synthetic compounds active in the central nervous system. Today, these compounds are produced both licitly and illicitly, have a wide range of biological targets, and produce numerous psychoactive effects in humans.

One class of compounds of interest is based on the β -phenylethylamine (PEA) scaffold. Structurally related to catecholamines (DA and NE), PEA is a trace-amine in the human brain with mild stimulant effects and is not considered a drug of abuse. Beginning in the mid-1970s, modern protocols of drug discrimination were established that made quantitative comparisons of the subjective effects of drugs using research animals (Overton 1991). These assays determine if a test drug is recognized in the same way as that of a training drug associated with a reinforcing reward, primarily using rat test subjects (Young 2009). In a resulting drug-discrimination paradigm, α -methyl PEA (amphetamine), 4-methoxy-N-methylamphetamine (PMMA), and 2,5-dimethoxy-4-methylamphetamine (DOM) were determined to be representative training compounds of the stimulant, empathogen (entactogen), and hallucinogenic drug classes, respectively (Glennon 1990; 1991; 1999; Glennon et al. 1997; Oberlender and Nichols 1988; Schechter and Rosecrans 1973). These three compounds are archetypes of their class and produce distinctive effects that cannot be generalized to other subjective effects by trained animals in drug discrimination assays. For example, rats trained to discriminate amphetamine will not recognize either PMMA or DOM, nor will rats trained on either DOM or PMMA recognize the other two classes (Glennon 1990; 1999; Oberlender and Nichols 1988). Drug discrimination studies are therefore a powerful tool for identifying and segregating test drugs into one of these drug classes.

The primary mechanism of action of amphetamine-like stimulants is the alteration of catecholaminergic signaling in the brain, where these drugs principally target the dopamine and norepinephrine transporters (DAT and NET). Although amphetamine acts as a potent neurotransmitter releaser at both transporters, most of its abuse related effects are attributed to its ability to augment DA levels in the mesolimbic reward pathway (Di Chiara and Imperato 1988; Negus and Miller 2014; Wise 1998).

Amphetamine is an FDA-approved prescription drug for the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy (Fry 1998; Mignot 1996; Seeman and Madras 1998). In addition, an extensive literature including clinical and pre-clinical studies suggests that amphetamine would be effective as a treatment for cocaine abuse (Banks et al. 2015; Grabowski et al. 2001; Mariani et al. 2012; Nuijten et al. 2016; Perez-Mana et al.

2011). However, the merits of using amphetamine as a cocaine treatment are limited by its own abuse potential (Negus and Henningfield 2015). Methamphetamine (N-methyl-amphetamine) is a strong stimulant that shares similar pharmacological properties with amphetamine and is FDA-approved as ADHD medication; however, it is heavily traded in the US clandestine drug market and is a detrimental drug of abuse (Mattson 2013). Cathinone (β -keto amphetamine or benzoylethanamine), which is the active component of the plant *Catha edulis* (khat), is another amphetamine-like stimulant that is consumed in the Middle East and Africa and is the only known naturally occurring amphetamine analog (Braenden 1979; Halbach 1979; Knoll 1979). In those regions where consumption is legal, khat leaves are traditionally chewed for their stimulant properties. In drug discrimination studies, cathinone can generalize in amphetamine trained rats (Kalix and Glennon 1986) and it can release ^3H -DA from preloaded brain slices (Kalix 1982). Similar to amphetamine, cathinone showed stereoselective DAT-mediated releasing and behavioral effects, with the S(–) enantiomer displaying more potent effects (Hutsell et al. 2016). Methcathinone, the N-methylated version of cathinone, was a popular drug of abuse in the former Soviet Union. It was first reported in Leningrad in the 1980s, and later it appeared in clandestine US drug markets, where it was classified as a Schedule I substance in 1992 (Dal Cason et al. 1997). Methcathinone is a more potent stimulant than cathinone, generalizes in animals trained to discriminate S(+)-amphetamine, and releases ^3H -DA from preloaded rat caudate nucleus (Glennon et al. 1987). As with other stimulants of the PEA family, the S enantiomer of methcathinone is more potent in behavioral and drug discrimination studies (Glennon et al. 1995).

Another recreational drug that has some structural similarity to amphetamine is 3,4-methylenedioxy methamphetamine (MDMA), which is known colloquially as ecstasy. Although worldwide production of amphetamine-type stimulants is dominated by amphetamine and methamphetamine production, MDMA abuse is still prevalent, especially among teenagers and young adults in Europe and the US (Aarde and Taffe 2017; UNODC 2017). In addition to its use as a recreational drug, MDMA has been studied as a potential support medication in the treatment of PTSD (Feduccia et al. 2018); however, it is currently classified as a Schedule I substance in the US. In drug discrimination studies, MDMA showed generalization in PMMA-trained rats and partial generalization in S(+)-amphetamine-trained rats, but is not recognized by DOM-trained rats. This suggests that MDMA is primarily an empathogen with some stimulant-like effects, but it is not a hallucinogenic agent (Glennon 1991; Glennon et al. 2007; Oberlender and Nichols 1988). These observations correlate well with subjective effects previously described in humans, where MDMA was reported as a distinctive entity from hallucinogens and “pure” stimulants (Oberlender and Nichols 1988). In contrast with amphetamine-like stimulants, MDMA is a releaser at all monoamine transporters (MATs), showing discrete preference for SERT and NET over DAT when tested *in vitro* using rat synaptosomes (Rothman and Baumann 2003). The administration of MDMA causes 5HT and DA release as measured by extracellular microdialysis of the dorsal striatum in rhesus monkeys and rats (Gudelsky and Nash 1996; Murnane et al. 2010). In self-administration studies and intracranial self-stimulation (ICSS) studies, MDMA displays less abuse liability than a pure stimulant such as S(+)-amphetamine or methamphetamine (Aarde and Taffe 2017). However, it has been suggested that chronic

use of MDMA can lead to the development of tolerance to the serotonergic effects, concomitant with a rise in DA-related effects, thereby causing increased abuse liability over time (Negus and Miller 2014).

Newer stimulant-like drugs confiscated in the illicit drug market are frequently advertised and sold as “legal-MDMA”. These formulations typically contain 4-substituted methcathinones such as mephedrone, methedrone, methylone, and flephedrone, among others (Baumann 2016; Glennon 2014; Hondebrink et al. 2018; Madras 2017). These drugs are often mixed with the extremely potent DAT inhibitor MDPV (Baumann 2016; Baumann et al. 2013b; Cameron et al. 2013; Glennon 2014; Glennon and Dukat 2017). These substituted cathinones emerged in illicit markets as a strategy for circumventing drug laws; new and unknown compounds are not subject to the same regulations as methamphetamine or MDMA. Initially, these compounds were sold either on the street or in convenience stores as “plant food” or “bath salt s” and were labeled as “not for human consumption” with the objective of distributing the recreational drugs as “legal highs” (Madras 2017). Authorities eventually identified these cathinone analogs as potential drugs of abuse and reclassified them as Schedule I substances, and their production and distribution is now prohibited (Baumann 2016). Drugs such as 4-methylmethcathinone (mephedrone) and MDPV were subsequently added to the list of controlled substances, but new unscheduled cathinone analogues periodically appear on the black market (Glennon and Dukat 2017).

Quantitative structure-activity relationship (qSAR) studies determined that the gradual transition from small to large volume substituents (-H < -F < -Cl < -CH₃ < -Br < -OCH₃ < -CF₃) in the 4-position of the aryl group of methcathinone is inversely correlated with DAT

over SERT selectivity (Sakloth et al. 2015), as measured in release assays using rat synaptosomes (Bonano et al. 2015; Sakloth et al. 2015). Similarly, the *in vivo* determination of DA and 5HT levels in the nucleus accumbens through microdialysis showed the same trend: higher selectivity for DA over 5HT release is inversely correlated with the volume of the substituent in the 4-position of methcathinone analogues (Suyama et al. 2016). The same correlation was observed in ICSS studies in rats where the abuse-related effects, mainly driven by DA, were also inversely correlated with the volume of the substituent in that position (Bonano et al. 2015). Together these data constitute compelling evidence that methcathinone, as with amphetamine and methamphetamine, is a selective DA releaser in both *in vitro* and *in vivo* assays. The selectivity of these compounds for DAT over SERT gradually decreases as the size of the 4-aryl substitution increases, which also decreases the abuse-related effects of these drugs. These studies demonstrate that *in vitro* pharmacological characterization of monoamine interactions with cathinones, and by extension amphetamines, is a valid strategy for studying and identifying drugs that could behave in manner similar to known drugs of abuse *in vivo*.

The appearance of “bath salts” and other analogs of known psychostimulants reflects a grander strategy on the part of illicit drug developers to produce “designer drugs”, which were formally known as “new psychoactive substances” (NPS). The motivation in producing these new compounds is to profit from the distribution of compounds that are not yet regulated by law. The United Nations Office of Drug and Crime reported the emergence of 739 NPS in the period between 2009 and 2016. The same office determined that about a quarter of a billion people (5% of the global adult population) used illicit drugs at least once in 2015, and that around 29.5 million of those individuals suffer from drug abuse disorders, which are defined as drug dependencies that require medical treatment (UNODC 2017). The periodic appearance of stimulant-class NPS will presumably continue and one of the many strategies for combating this public health hazard is to anticipate which new molecules will appear on the clandestine market. This information can effectively warn authorities about potential new compounds and can expedite the application of appropriate measures. As discussed above, qSAR studies of compounds derived from amphetamine or cathinone scaffolds are a powerful tool for identifying potential new drugs that have significant DAT and SERT activity. Although the methods for profiling MAT ligands have been utilized for more than a decade, no major innovation has been implemented to expedite this endeavor. Here we will discuss a new emerging approach for studying the effects of molecules at MATs in a cell-based assay and how this method constitutes a robust new strategy for expediting functional characterization of new compounds.

Ion channel-like behavior in monoamine transporters

Monoamine transporters are comprised of three different genes, SLC6A3, SLC6A4 and SLC6A2, that encode the DAT, SERT and NET, respectively. These are integral membrane proteins involved in controlling the extracellular concentration of the biogenic amines DA, 5HT and NE. These transporters are part of a large super family of solute carriers and are Na⁺- and Cl⁻-dependent symporters. Early studies using SERT-expressing platelets demonstrated that Na⁺ is required for 5HT transport (Rudnick 1977). Later, a fixed-stoichiometry model was proposed for SERT operation (Rudnick and Nelson 1978). In this

model, positively charged Na^+ and 5HT (5HT is positively charged at physiological pH) and one negatively charged Cl^- bind to the transporter in an “outward facing” conformation. The transporter then undergoes a conformational change to an “inward facing” conformation and the ions and 5HT are released to the intracellular space. In the last step, the empty inward facing transporter binds K^+ , which facilitates the transporter’s return to the initial outward facing conformation where K^+ is released to the extracellular space (Rudnick and Nelson 1978). In this secondary active transport mechanism, the Na^+ electrochemical gradient supplies the free energy required to concentrate 5HT inside cells. According to this model, the net movement of charge per cycle is zero, implying that SERT’s full transport cycle is electroneutral. Biochemical studies measuring cysteine accessibility, intramolecular FRET (Androutsellis-Theotokis and Rudnick 2002; Henry et al. 2003; Loland et al. 2004; Zhao et al. 2011), and later crystal structures of the bacterial leucine transporter LeuT (a bacterial ortholog of mammalian MATs) provided new insights about ligand binding and some of the structural transitions within the transport cycle (Kazmier et al. 2014; Penmatsa and Gouaux 2014).

When SERT cDNA became available, experiments that tested the electrical properties of 5HT transport were performed. SERT was expressed in a heterologous expression system (*Xenopus laevis* oocytes) and the experiments revealed three distinct ionic conductances: 1) a constitutive leak current in the absence of organic substrate, 2) a transient current resulting from brief changes in membrane electrical potential, and 3) an inward current induced by exposure to substrates (Mager et al. 1994). The assumption before that time was that the transport cycle was electroneutral and the measurement of the 5HT-induced current therefore challenged that view. Moreover, 5HT transport was previously determined to be independent of membrane potential (Mager et al. 1994; Rudnick and Nelson 1978), but the 5HT-induced current showed a marked voltage dependence, suggesting that the ionic current measured is either uncoupled to 5HT transport or that the stoichiometry of the transport cycle is variable (Mager et al. 1994; Su et al. 1996). Transport of substrates through DAT and NET following the classical alternating access/fixed stoichiometry model have a different stoichiometry than that of the transport stoichiometry of SERT; DAT transports two Na^+ per cycle and neither DAT nor NET counter-transport K^+ (Gu et al. 1994), rendering the cycle electrogenic. Electrophysiological studies in parallel with uptake studies showed that for DAT and NET the substrate transport and the substrate-induced currents are voltage dependent but the amount of ionic charge per substrate measured during transport is several folds larger than what would be predicted by the fixed-stoichiometry model (Galli et al. 1996; Galli et al. 1995; Sonders et al. 1997). Furthermore, single-channel like events exceed the magnitude of single charge events predicted from the classical model (Carvelli et al. 2004; Galli et al. 1996; Lin et al. 1996). These results clearly showed that the alternating-access/fixed-stoichiometry model for the operation of MATs is an over-simplification, and alternative hypotheses were developed to refine models of the transport mechanism of these transporters (De Felice 2016). Using theories developed for ion channels, Su et al. (Su et al. 1996) first developed a single-file multi-transport model, assuming 3 binding sites and two moving species. This model can account for experimentally observed steady-state currents observed as a function of $[\text{Na}^+]$ and $[\text{5HT}]$, the leak current, and the variable stoichiometry of SERT (Mager et al. 1994; Su et al. 1996). De Felice et al. went one step further proposing

that the coupling between the organic substrate (5HT) and Na⁺ uses single-file diffusion through a long and narrow pore (DeFelice et al. 2001). This new ion channel-like model was able to reconcile several of the inconsistencies between the alternating access model and experimental data (Adams and DeFelice 2002; 2003). Using a fast perfusion system, transporter currents associated with exposure to substrates were studied with detailed time resolution in mammalian cells (HEK293) expressing MATs. When these cells are under voltage-clamp and exposed to a substrate, a sharp inward peak-current first appears, which then rapidly decays to a steady-state level that deactivates and returns to baseline when the substrate is washed out (Erreger et al. 2008; Schicker et al. 2012). When cells expressing DAT or SERT are exposed to substrates, the resulting inward currents have the same graphical kinetic shape. While DAT currents were modeled using a pure multi-state alternating access kinetic model, and SERT currents were modeled using a similar model plus an uncoupled ion channel state, both models fit the data reasonably well. It has been posited that the rapid-onset peak-current is capacitive in nature and represents the binding of molecular charges (substrates and ions) to the transporter, which is immersed in the membrane electrical field. Meanwhile, the steady-state current is a rheogenic conductance. For detailed discussions see (Erreger et al. 2008) and (Schicker et al. 2012). Although these models provide an invaluable analytical tool, a definitive model for MAT operation is still not settled, but it is widely accepted that MAT operation occurs with inward ionic currents that can depolarize the plasma membrane. Indeed, substrates of DAT can increase the electrical excitability of dopaminergic neurons through a mechanism that involves DAT current-mediated depolarization (Branch and Beckstead 2012; Ingram et al. 2002). As mentioned before, DAT substrates such as amphetamine are also releasers of DA, and the augmentation DA levels in the brain can stimulate inhibitory autoreceptors, thereby hyperpolarizing cells and shutting down action potential firing. Therefore, from an electrophysiological perspective, DAT substrates have a dual effect: at low concentrations they increase neuronal firing (direct DAT effect) and at high concentrations they can decrease it (activation of autoreceptors) (Branch and Beckstead 2012; Ingram et al. 2002). In contrast, inhibitors of MATs have the opposite electrical effect through the blockade of constitutive inward “leak” currents, which results in membrane hyperpolarization (Mager et al. 1994; Sonders et al. 1997).

Electro-engineering of cells to develop biosensors to sense monoamine transporter activity

In the 1950s, seminal studies conducted by Hodgkin and Huxley described the ionic currents responsible for the propagation of action potentials in the squid axon and elucidated what would become the basis of modern biophysics. Their research led to the identification, characterization, and classification of ion channels—integral membrane proteins that linked biophysics to other disciplines such as neuroscience and neuropharmacology. In the late 1980s, advances in molecular biology permitted the cloning and expression of proteins of interest in cells. Immortalized cell lines or *Xenopus* oocytes expressing ion channels allowed for a detailed characterization of ion channel function, including mechanisms of permeation and gating. Overexpression of a specific channel in these cells improved detection of their electrical signals that might otherwise be difficult to achieve in native systems. Additionally,

channels can be co-expressed with accessory subunits or partner proteins for studying higher-level regulation, for detailed information about ion channels refer to (Hille 2001). We wanted to take the information available about specific ion channels and their electrical properties in expression systems and use that information to make these systems responsive to other biological phenomena. Ideally, we sought to develop cells expressing ion channels that could function as novel biosensors.

Ion channels are divided into two main super families: those that respond to chemical signals (ligand-gated channels) and those that are responsive to fluctuations of the membrane electrical potential (voltage-gated channels) (Hille 2001). An important subgroup of channels that are a part of the latter super family are voltage-gated Ca^{2+} channels, which are channels that selectively allow some bivalent ions such as Ca^{2+} and Ba^{2+} to permeate into cells. Voltage-gated Ca^{2+} channels are important in excitable cells because they can transduce an electrical signal in the plasma membrane (e.g. action potential) into changes in cytosolic Ca^{2+} signals (Catterall et al. 2005). Ca^{2+} is an archetypical second messenger. In quiescence, the intracellular Ca^{2+} concentration is very low (~100 nM), but after cell activation and channel opening, the Ca^{2+} concentration can increase globally to between 10 and 100-fold the resting level, and proximal to the Ca^{2+} channel nanodomain it can reach even higher concentrations (Eggermann et al. 2011; Tadross et al. 2013). The extracellular concentration of Ca^{2+} is normally around 1 to 2 mM, resulting in an enormous electrochemical gradient (~10,000 times more concentrated than inside the cell). This in combination with a negative plasma membrane potential, results in a strong inward driving force for Ca^{2+} entry into the cell upon channel activation or opening. Therefore, when Ca^{2+} channels open under physiological conditions, Ca^{2+} enters the cell along a steep electrochemical gradient that can't equilibrate to the ion's reversal potential (Berridge 2006; Berridge et al. 2003; Eggermann et al. 2011; Hille 2001).

The intracellular Ca^{2+} concentration in cells at rest is primarily defined by the efflux of Ca^{2+} through the high affinity, low capacity plasma membrane Ca^{2+} ATPase (PMCA), and to a lesser extent, exchangers that counteract Ca^{2+} "leak" into the cell by channels at the plasma membrane (Brini and Carafoli 2011). At the steady-state, resting conditions, the net flux of Ca^{2+} through the plasma membrane and membranes of intracellular organelles is zero (Rios 2010). Upon depolarization, an influx of Ca^{2+} through plasma membrane associated voltage-gated Ca^{2+} channels increases the intracellular Ca^{2+} concentration as a function of time. Most voltage-gated Ca^{2+} channels show, to some extent, voltage and Ca^{2+} dependent inactivation that limits the population of Ca^{2+} channels opened at the steady-state of a depolarization event (Catterall 2011). As the intracellular Ca^{2+} concentration rises, activation of the low affinity, high capacity $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) balances influxes through channels, thereby contributing to a new steady state in which the resting Ca^{2+} level is elevated. Repolarization of the membrane shifts the population of open-state Ca^{2+} channels to a predominantly closed-state population, which decreases the net influx of Ca^{2+} into the cell. Finally, proportionally elevated Ca^{2+} efflux through NCX then decreases the intracellular Ca^{2+} concentration to the initial resting state (Brini and Carafoli 2011). Thus, at rest, when most channels are closed, the influx of Ca^{2+} is low and the intracellular Ca^{2+} concentration is limited by high affinity extruding mechanisms. During channel activation, however, the intracellular Ca^{2+} concentration is proportional to the number of open Ca^{2+}

channels, which will depend on the membrane potential in the case of voltage-gated Ca^{2+} channels (Fig. 1).

Given the many types of voltage-gated Ca^{2+} channels that display a variety of biophysical properties including different activation and inactivation voltage dependency and kinetics, it is possible to devise a system in which voltage-gated Ca^{2+} channels work as a reporter of membrane depolarization evoked by MAT activity. In principle, the co-expression of MATs with a voltage-gated Ca^{2+} channel with the right voltage sensitivity and kinetics could produce a system in which extracellular MAT substrates can modulate the intracellular Ca^{2+} concentration in a dose-dependent manner. The first instance of such a system was demonstrated using a cultured skeletal muscle cells. Muscle cells have tight control of the intracellular Ca^{2+} level as function of membrane potential: they work as a natural voltmeter. When cultured muscle cells are exposed to brief pulses of isosmotic external solutions with increasing K^+ concentrations, their membranes depolarize in a Nernstian manner and produce dose-dependent Ca^{2+} signals (Eltit et al. 2014; Jaimovich and Rojas 1994). Similarly, simultaneous recordings of membrane potential and intracellular Ca^{2+} levels showed a sigmoidal relationship between membrane potential and the intracellular Ca^{2+} signal amplitude (Eltit et al. 2011; Garcia and Beam 1994). The voltage sensor that mediates Ca^{2+} release in skeletal muscle cells is the L-type Ca^{2+} channel $\text{Ca}_v1.1$. The physical interaction between $\text{Ca}_v1.1$ and RyR1 confers an additional amplification of the Ca^{2+} signal and mediates the release of Ca^{2+} from internal stores. Muscle cells do not express MATs and exposing these cells to 5HT or S(+)-MDMA did not produce any Ca^{2+} signals. However, a SERT-expressing muscle cell line generated using a retroviral vector did respond to the exposure of known SERT substrates, generating instantaneous Ca^{2+} signals that return to the baseline after washout (Ruchala et al. 2014). The SERT inhibitor fluoxetine blocks these Ca^{2+} signals, while, conversely, the voltage-gated Na^+ channel blocker tetrodotoxin does not affect the progression of the Ca^{2+} signal (Ruchala et al. 2014). These results were the first indication that the substrate-activated and SERT-mediated depolarization is sufficient for L-type Ca^{2+} channel activation. Additionally, cultured $\text{Ca}_v1.1$ -null skeletal muscle cells do not produce SERT substrate-induced calcium signals after viral transduction of SERT cDNA, further implicating L-type Ca^{2+} channel participation. Interestingly, these cells display robust caffeine-induced Ca^{2+} signals, suggesting direct activation of RyR1 (Ruchala et al. 2014). This set of experiments strongly supports the idea that SERT-mediated currents can depolarize the plasma membrane and activate L-type Ca^{2+} channels.

Further evidence for the unidirectional signaling from MATs to voltage-gated Ca^{2+} channels came from experiments on HEK293 cells co-expressing SERT or DAT and L-type Ca^{2+} channels (Cameron et al. 2015; Ruchala et al. 2014). This cell line has several advantages as the cells have no background expression of voltage-gated Ca^{2+} channels, do not endogenously express MATs, and are easy to transfect with high yield. These cells do not produce Ca^{2+} signals when expressing either SERT, DAT or L-type Ca^{2+} channels alone and are exposed to MAT substrates, providing a clean background for co-expression experiments (Cameron et al. 2015; Ruchala et al. 2014). First, the voltage sensitivities of $\text{Ca}_v1.2$, $\text{Ca}_v1.3$ and $\text{Ca}_v2.2$ were tested in combination with the transfection of the auxiliary subunits β_3 and $\alpha_2\delta$ using whole-cell voltage-clamp (Cameron et al. 2015; Ruchala et al. 2014). The threshold for voltage activation was consistent with previous observations that found that

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Ca_v1.3 is activated the most at more negative potentials followed by Ca_v1.2, and then Ca_v2.2 which requires the strongest depolarizations for activation of the three (Cameron et al. 2015). Fascinatingly, when DAT or SERT were co-expressed with Ca_v1.3 or Ca_v1.2 Ca²⁺ channels in these cells, they produced Ca²⁺ signals upon exposure to MAT substrates (Cameron et al. 2015; Ruchala et al. 2014). In contrast, Ca_v2.2 (N-type) co-expressed with DAT or SERT was refractory to MAT substrate exposure (Cameron et al. 2015; Ruchala et al. 2014). These results provide a rough estimation of the minimum depolarization that substrates of MAT produce in unclamped, intact cells. For example, in these experimental conditions, a substrate-induced DAT current should depolarize the membrane at least ~20 mV (assuming a resting membrane potential of ~-50 mV) for the activation of Ca_v1.3 and Ca_v1.2, but not Ca_v2.2 (Cameron et al. 2015).

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Additional evidence that supports the idea of electrical crosstalk from MATs to L-type Ca²⁺ channels is that both isradipine (potent L-type Ca²⁺ inhibitor) and MDPV (strong DAT inhibitor) suppress the Ca²⁺ signal induced by either DA or amphetamine in cells co-expressing L-type Ca²⁺ channels and DAT (Cameron et al. 2015). In addition, exposing the co-expressing cells to Li⁺ results in a large Ca²⁺ signal that is absent in cells expressing only L-type Ca²⁺ channels (Cameron et al. 2015). MATs have a constitutive conductance to Li⁺ in the absence of substrates (Borre et al. 2014; Lin et al. 1996; Mager et al. 1994; Sonders et al. 1997), and the Ca²⁺ signals measured under extracellular Na⁺ to Li⁺ replacement strongly indicate that the current through the transporter is mediating the depolarization that activates Ca²⁺ channels.

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The previous experiments provide evidence for a model in which substrates of MATs can induce inward currents through the transporter, thereby depolarizing the cell membrane in a range that is sufficient for the activation of L-type Ca²⁺ channels (Fig. 1). Superimposition of dose-response curves for the DAT-mediated inward current (voltage-clamp experiments), Ca²⁺ signals (imaging data in intact cells), and ³H-DA uptake showed that the DAT current starts to appear at lower DA concentrations than the Ca²⁺ signal (Fig. 2). Additionally, the DA uptake is relegated to even higher DA concentrations (Cameron et al. 2015; Ingram et al. 2002). The slope of the curve for the Ca²⁺ signal is steeper than the slope of the curve of the DA-induced current (Fig. 2). This is consistent with a positively cooperative system in which the initial depolarization mediated by DAT conductance activates the depolarizing Ca²⁺ conductance, resulting in a positive feedback loop that potentiates the Ca²⁺ signal.

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S(-)-methcathinone (EC₅₀=14.8± 0.4 nM) is many times more potent than DA (EC₅₀=86.9± 9.7 nM) in a synaptosome release-assay, and is one of the most potent releasers at DAT (Rothman and Baumann 2003; Rothman et al. 2003). We measured the potency of S(-)-methcathinone and DA in producing Ca²⁺ signals in cells expressing DAT and the voltage-gated Ca_v1.2 channels and reproduced the differences in potency between these two compounds (EC₅₀^{S(-)MCAT}=77±7 nM; EC₅₀^{DA}=1,302±109 nM) (Fig. 3). We had previously reported that S(+)-amphetamine, which is known to be a stronger releaser than DA, had an EC₅₀ of 144 nM in our Ca²⁺-assay (Cameron et al. 2015). These results suggested that the Ca²⁺ assay cannot only identify releasers, but it can also determine potencies with good correlation to synaptosome release data. Two relevant DAT blockers with different potencies are MDPV and cocaine. Previously, ³H-DA uptake competition

using cocaine or MDPV in DAT expressing HEK-293 cells gave IC_{50} values of 995 nM and 28 nM, respectively (Cameron et al. 2013). When tested in our DAT and $Ca_v1.2$ co-expressing Ca^{2+} assay, these compounds alone do not produce signals, but do competitively block DA-induced Ca^{2+} signals in a dose-dependent manner ($IC_{50}^{Cocaine}=1,582\pm115$ nM; $IC_{50}^{MDPV}=35\pm3$ nM) (Fig. 3). These experiments further demonstrate that this Ca^{2+} -assay can be used to differentiate substrates and blockers of the transporters and determine drug potencies with good correlations to those determined by other assays.

To revisit the notion of biosensors, our assay's highly reproducible Ca^{2+} signals, which are generated by MAT substrates or inhibited by MAT blockers, offer an unprecedented opportunity for the pharmacological characterization of novel compounds at DAT, SERT or NET using live cell Ca^{2+} imaging.

Validation of Ca^{2+} -based biosensors to characterize ligands of monoamine transporters.

The most widely accepted techniques for studying MAT ligands *in vitro* are brain-derived synaptosome uptake and release assays (Rothman and Baumann 2003; 2006; Rothman et al. 2000). These are radioligand-based techniques in which synaptosomes are isolated from the brains of rats. Synaptosomes are prepared by homogenization of the rat caudate for DAT experiments, the whole brain minus the cerebellum for SERT experiments, and the whole brain minus the caudate for NET experiments. In the uptake-assay, synaptosomes are exposed to tritiated versions of the neurotransmitter corresponding to the MAT of interest (3H -5HT, 3H -DA or 3H -NE). In a given experiment, specific blockers of the two MATs not being investigated are applied in the presence of varying concentrations of a test compound against the MAT of interest (Rothman et al. 1993; Rothman et al. 2000). Competitive uptake proceeds for 30 minutes, after which synaptosomes are collected by fast-filtration, washed once, and retained for tritium measurement in a scintillation counter. Although appreciable uptake inhibition activity is a good indication that a compound is active at a transporter, it is not enough to determine whether the test drug is a substrate or a blocker, as both types of compounds will compete with radiolabeled neurotransmitter uptake. A fundamental difference between MAT substrates and blockers is that substrates can promote neurotransmitter reverse transport, causing efflux of the intracellular neurotransmitter. Conversely, blockers act as a “cork in a bottle” and inhibit the MAT uptake and efflux cycles. Rothman, Baumann and collaborators developed a method that used synaptosomes to discriminate MAT substrates (releasers) and inhibitors (blockers) (Rothman and Baumann 2003; Rothman et al. 2000). In this method synaptosomes are pre-loaded with tritiated substrates: 3H -5HT for the study of SERT-mediated release and 3H -MPP⁺ for the study of DAT- or NET-mediated release (Solis et al. 2017). The preloaded synaptosomes are exposed to the test drug and retained radioactivity is measured in the scintillation counter. If a test drug is a substrate (releaser) it will induce the efflux of the radio-labeled substrate, decreasing the retained counts in a dose-dependent manner. Released radio-counts are normalized to the maximal release, which is elicited by tyramine—a strong releaser. One limitation of the release assay is that synaptosomes loaded with a radioactive substrate show a nonspecific radioactive leak that is to some extent counteracted by MAT activity; however,

in the presence of a strong blocker, this counteracting transport is inhibited, and the radioactive leak becomes apparent. Thus, blockers of MATs display an apparent release, sometimes also called pseudo-release, which is much smaller in magnitude than the release induced by substrates (Baumann et al. 2013b; Solis et al. 2017). Most strong substrates, like methamphetamine (DAT) or MDMA (SERT), display full release, while other substrates like 4-methyl-MA at DAT produce partial release (Solis et al. 2017), which is defined as release efficacy larger than the pseudo-release caused by blockers but less than that of the full release caused by tyramine (Rothman et al. 2012; Sandtner et al. 2016). The potential for the determination that a compound is a weak, partial releaser therefore introduces some uncertainty as to how these compounds function mechanistically.

We sought to develop a parallel method for distinguishing substrates from blockers, but rather than relying on MAT transport mechanisms, our method was designed to take advantage of MAT electrical properties. As previously mentioned, transporter substrates and blockers produce opposite electrical signals on MAT-expressing cells: substrates depolarize the cell membrane while blockers hyperpolarize it. This fundamental difference can be used to track the effect of potential new drugs at the transporter level. Traditionally, electrophysiological measurements in cells required the use of microelectrodes, amplifiers and skillful operators that would measure the ionic current amplitude necessary to hold an imposed electrical potential constant across the intracellular and extracellular compartments of the cell. This voltage-clamp technique can provide details about the electric and kinetic properties of ionic currents. One disadvantage to voltage clamping MATs is that their electrical currents are small in comparison to traditional ion channels. Although these sophisticated experiments have indeed been used to characterize compounds at transporters, it is not the ideal methodological choice for extensive pharmacological studies. The direct measurement of electrical currents by voltage-clamp is extremely low throughput while medicinal chemistry studies of MAT-ligand relationships (e.g. structure-activity studies) require the assessment of tens to hundreds or even thousands of compounds. Thus, the use of voltage-gated Ca^{2+} channels as sensors of the electrical activity of transporters—through intracellular Ca^{2+} measurement using live-cell fluorometry—can increase MAT/ligand measurement throughput enough to be compatible with pharmacological investigations.

We introduced this new methodology in the preclinical characterization of amphetamine variants. Amphetamine is a primary amine with higher potency as a releasing agent at NET and DAT than at SERT (Rothman and Baumann 2003). Interestingly, a methyl substitution in the 4th position on its phenyl ring (4-methylamphetamine or 4-MA) makes it a potent but nonselective releaser at all three transporters (Baumann et al. 2011). Other abused drugs of the PEA family with similar profiles that are classified as Schedule I compounds in the US are MDMA and mephedrone (Baumann et al. 2013a). As with 4-MA, MDMA and mephedrone have a 4-substituted phenyl ring, but in contrast, they are secondary amines. Thus, it is possible that the N-alkyl modification of 4-MA would not significantly alter its activity at all three transporters. 4-MA is a street drug reported in the US and Europe and it can cause severe intoxications and potentially even fatalities. Exploring modifications on this scaffold that retain activity at MATs, along with investigating the abuse-related effects of these compounds in animals can not only provide new insights about drug-transporter

interactions, but also serve to warn authorities and the scientific community of potentially emergent NPS before they appear in the clandestine market.

4-Methylamphetamine (4-MA)

R

$\text{CH}_3 = \text{N-methyl 4-MA}$

$\text{CH}_2\text{CH}_3 = \text{N-ethyl 4-MA}$

$(\text{CH}_2)_2\text{CH}_3 = \text{N-propyl 4-MA}$

$(\text{CH}_2)_3\text{CH}_3 = \text{N-butyl 4-MA}$

In order to further investigate the behavior of secondary amine 4-MA analogues at MATs and their abuse related effects in animals, four N-alkyl substituted 4-MA compounds were synthesized: N-methyl-, N-ethyl-, N-propyl and N-butyl-4-MA. In competition studies using synaptosomes, these compounds were active at all three MATs but showed decreased potency as the N-alkyl chain was extended, where N-butyl-4-MA was basically inactive (Solis et al. 2017). In the synaptosome-release assay, N-methyl-4-MA was a full releaser at all three transporters. Accordingly, this drug produces inward currents in *Xenopus* oocytes expressing DAT or SERT (NET is recalcitrant to expression in *Xenopus* oocytes). In the Ca^{2+} assay, N-methyl-4-MA produced Ca^{2+} signals in cells expressing each of the transporters, indicating that N-methyl-4-MA is a releaser (or substrate) at the three MATs (Solis et al. 2017). As expected, S(+) and racemic N-methyl-4-MA showed potent release activity at all three MATs as measured in synaptosomes and in the Ca^{2+} assay (Battisti et al. 2018). To assess the abuse-related effects of this drug *in vivo*, it was tested in ICSS studies. S(+)N-methyl-4-MA produced potentiation of ICSS at low stimulation frequencies, which is characteristic of drugs that increase DA in the brain, and it also produced depression of ICSS at high stimulation frequencies, an effect that, although somewhat less specific, is associated with increases of 5HT (Battisti et al. 2018; Negus and Miller 2014). For reference, MDMA is also nonspecific MAT releaser and shows similar biphasic results in ICSS studies (Bauer et al. 2013; Negus and Miller 2014). R(–)N-methyl-4-MA is an uncharacteristic MAT substrate in that it is a releaser at all three transporters and has a strong preference for NET

over SERT but is quite weak at DAT as measured in synaptosomes and the Ca^{2+} -assay. In ICSS studies, this compound shows no facilitation at low stimulation frequencies but retains the ability to depress ICSS at higher stimulation frequencies. This result demonstrates that the abuse-related effect of racemic N-methyl-4-MA, as evidenced by ICSS facilitation, resides in the S(+) enantiomer as it has the strongest DAT effect (Battisti et al. 2018; Solis et al. 2017).

The racemate of N-ethyl-4-MA as well as both enantiomers produced full release at SERT when tested in synaptosomes and produced Ca^{2+} signals when tested using the Ca^{2+} assay, where the S(+) enantiomer was more potent than R(–) in both assays. When N-ethyl-4-MA was tested in the synaptosome-release assay, it behaved as a partial releaser at DAT, showing an efficacy of about 50% of the maximal release (Battisti et al. 2018; Solis et al. 2017). Unexpectedly, this compound showed an outward deflection in the current trace in oocytes expressing DAT, which is characteristic of blocker action at the transporters (Solis et al. 2017). In addition, in the Ca^{2+} assay this compound did not produce Ca^{2+} signals at any concentration but did block the DA-induced Ca^{2+} signal in a dose-dependent manner (Solis et al. 2017). These results suggested that some partial MAT releasers function as blockers in electrophysiological techniques. These results were obtained using the racemate, and since it is possible that differential effects of the two stereoisomers could explain the partial release effect of N-ethyl-4-MA at DAT, the two enantiomers of N-ethyl-4-MA were synthesized and tested in the synaptosome-release and Ca^{2+} -assays. In both assays, S(+)N-ethyl-4-MA was much more potent than R(–)N-ethyl-4-MA (Battisti et al. 2018). Additionally, in the synaptosome release assay, both enantiomers were shown to be partial releasers (~50% of the maximal release) while in the Ca^{2+} -assay both enantiomers were shown to be blockers (Battisti et al. 2018). Accordingly, racemic N-ethyl-4-MA and the S(+) enantiomer alone showed marginal facilitation in ICSS, while the R(–) enantiomer, which is very weak at DAT, showed no facilitation. This clearly demonstrated that DAT releaser activity is necessary for maintaining facilitation in ICSS at low frequencies, which is a measure of abuse potential (Battisti et al. 2018; Solis et al. 2017). N-ethyl-4-MA and its enantiomers showed depression of ICSS at higher stimulation frequencies, which correlates well with their releaser activity at SERT (Battisti et al. 2018; Solis et al. 2017). Furthermore, these results demonstrate that partial release is not a result of the opposite effects of enantiomers, but rather it is a property intrinsic to the compound-transporter interaction.

At NET, N-ethyl-4-MA behaved as a full releaser in the synaptosome-release assay and, as expected, in the Ca^{2+} -release assay this compound was a depolarizing agent and induced Ca^{2+} signals (Solis et al. 2017). When enantiomers of N-ethyl-4-MA were tested at NET in synaptosomes, both were full releasers, whereas in the Ca^{2+} assay, neither S(+)N-ethyl-4-MA nor R(–)N-ethyl-4-MA was fully efficacious in eliciting Ca^{2+} signals (~60% and ~35% of the signal induced by NE, respectively) (Battisti et al. 2018). These results suggested that drugs that generate depolarizing currents, even if not completely efficacious, might function as full releasers in the synaptosome assay.

N-propyl-4-MA promoted full release in synaptosomes at SERT, elicited inward currents in SERT-expressing oocytes, and evoked Ca^{2+} signals to the level of 5HT controls in a dose-dependent manner in HEK-293 cells expressing SERT and Ca^{2+} channels (Solis et al. 2017).

These results strongly suggested that is a substrate at SERT. At DAT, N-propyl-4-MA did not produce release in synaptosomes, and caused an outward current in oocytes. Accordingly, it did not elicit Ca^{2+} signals, but did block the DA-induced Ca^{2+} signal with low potency (Solis et al. 2017). N-propyl-4-MA produced partial release (about 50% of the maximal release) at NET when tested in synaptosomes. It did not produce Ca^{2+} signals in cells co-expressing NET and Ca^{2+} channels but blocked the signal induced by NE (Solis et al. 2017). Similar to N-ethyl-4-MA at DAT, N-propyl-4-MA at NET is a partial releaser and not a depolarizing agent, suggesting that partial release can happen in the absence of the inward current of the transporter (Solis et al. 2017). When tested in ICSS, N-propyl-4-MA did not produce facilitation consistent with the absence of release activity at DAT, but did produce low potency ICSS depression at higher frequencies, which fits with its low potency at SERT as a releasing agent (Solis et al. 2017).

A prevailing trend of MAT-ligand interactions is that small compounds that work as substrates will become blockers as their overall size increases. Thus, it is expected that the incremental substitutions of N-alkyl 4-MA described above would morph the activity of the parent compound of the series from a substrate to a blocker (Solis et al. 2017). The important finding here is that the transition point from substrates to blockers is not the same at each transporter. For example, N-methyl-, N-ethyl- and N-propyl- 4-MA are substrates and full releasing agents at SERT, but of these three, only N-methyl is a substrate at DAT, while N-ethyl is a blocker that caused partial release and N-propyl is a blocker that caused no release at DAT (Solis et al. 2017). These findings show that these agents are good examples of hybrid compounds that have differential effects at each of the MATs (Blough et al. 2014). In addition, as evidenced by N-ethyl-4-MA at DAT (partial release and no current) and N-ethyl-4-MA at NET (full release and partial current), these findings show that the transition from a substrate to a blocker is a continuum in which intermediate compounds can display intermediate behaviors.

These experiments clearly showed that the new Ca^{2+} -assay can be used to assess the effects of test drugs at MATs with good qualitative correlation to the synaptosome-assay results, but it was not clear to what extent the quantitative results correlated with values obtained using synaptosomes. To address this issue, linear correlation analysis was performed to compare the potencies of drugs as tested in both assays (Battisti et al. 2018; Solis et al. 2017). Here, we provide a new linear correlation analysis comparing the potencies of several drugs that we have tested using the Ca^{2+} assay with their potencies as determined by the synaptosome assay and previously reported in the literature (Fig. 4). For consistency, IC_{50} values of compounds that were determined to be blockers in the Ca^{2+} assays are correlated to IC_{50} values of uptake inhibition from the synaptosome-uptake assay, while EC_{50} values for producing Ca^{2+} signals in HEK293 cells are correlated to EC_{50} values for promoting release in the synaptosome-release assay. The values used to produce these correlations are depicted in Table 1. This analysis must be interpreted with some caution since the data have been collected from several different sources and the variability between experimental conditions introduces some uncertainty. The effects of twenty-one transporter-drug conditions were included in the correlation of releasers, yielding a linear correlation coefficient of 0.73 and a slope of 1.04 ± 0.15 ($p < 0.0001$). Fourteen transporter-drug conditions were included for the blocker correlation, which has a linear correlation coefficient of 0.93 and a slope of 0.99

± 0.08 ($p < 0.0001$) (Fig. 4). These strong positive correlations between the values produced by the two assays clearly demonstrate that the electrical characterization of compounds using the Ca^{2+} -assay can provide quantitative information about the potency of drugs akin to what the synaptosome assays can provide. Emblematic drugs of abuse such as S(-)methcathinone and S(+)-amphetamine were determined to have potent DAT activity as substrates in both assays, and both assays were able to distinguish the difference in the potencies of two relevant DAT blockers, MDPV and cocaine (Fig. 4). Interestingly, R(-)-N-ethyl-4-MA is a weak but fully efficacious releaser at NET in the synaptosome-release assay, but it is not efficacious in producing signals in the Ca^{2+} assay (less than ~35% of the control) (Battisti et al. 2018). In the correlation of releasers (Fig. 4) this compound-transporter condition appears as an outlier, most likely due to the overestimation of the potency in the Ca^{2+} assay because its low efficacy caused an apparent shift of its potency value to the left. Another compound-transporter condition that is a slight outlier on the inhibitor linear regression curve is R(-)-N-propyl-4-MA at SERT (the only SERT inhibitor tested, Fig. 4). This drug is weak but fully efficacious in blocking uptake in synaptosomes, but in the Ca^{2+} -assay only blocked 60% of the total signal induced by 5HT (Battisti et al. 2018). This decrease in efficacy in the Ca^{2+} -assay may also overstate the potency of the drug. Thus, drugs that are in the boundary between substrates and blockers at a given MAT might slightly deviate from the ideal regression curve, but the majority of the drugs tested behave proportionally in the synaptosome and Ca^{2+} assays.

The voltage-gated Ca^{2+} -assay described above is a new addition to a number of available techniques for characterizing certain drugs' effects at MATs. Together with the synaptosome-based efflux and uptake-competition approaches, this Ca^{2+} -assay is one of only a few available techniques that can reliably distinguish substrates from blockers while simultaneously determining the potencies of compounds at MATs. As mentioned before, these techniques are preferable when many compounds need to be screened, in addition they can provide preliminary *in vitro* profiling at transporters and help discriminating active from inactive drugs. Active compounds can then be studied *in vivo* by neurochemical techniques such as microdialysis (Baumann et al. 2011) or fast scan cyclic amperometry (Hoffman et al. 2016; John and Jones 2007; Siciliano et al. 2014; Siciliano et al. 2018) or characterized in behavioral studies such as ICCS (Negus and Miller 2014). Another advantage of the Ca^{2+} -assay is that it does not require the use of radioactively labeled ligands that are typically expensive and whose use must adhere to special institutional regulations. Additionally, since this method uses cultured cell lines, it eliminates the use of experimental animals and thereby minimizes bioethical concerns and the consideration of additional regulations.

Currently, several methods are available for measuring intracellular Ca^{2+} signals. A variety of bright, commercially available, and membrane-permeable Ca^{2+} sensors can be used to measure these signals by fluorescence microscopy or using optimized fluorescence plate readers. The main limitation of this new technique is that because the Ca^{2+} signal is an indirect measure of MAT activity, non-specific effects of drugs on other proteins that affect cellular Ca^{2+} homeostasis could make some results unreliable. In general, we have not yet encountered non-specific drug effects at physiologically relevant concentrations; if they occur, non-specific effects are unmasked at irrelevantly high concentrations of test drugs. Importantly, non-specific signals are detectable by control experiments with cells that do not

express transporters or Ca^{2+} channels and are kinetically much slower than specific signals, which have a faster onset. This new Ca^{2+} -based technique for assessing the activity of MAT ligands is still incipient but future optimizations will continue to improve the measurement and assessment of ligand-transporter interactions.

In summary, the development of this new Ca^{2+} -based assay serves to meet the need for profiling the effects of novel compounds at MATs. The application of this new technology can expedite the identification of amphetamines and cathinone variants that have equal or higher activity at DAT, NET or SERT. In combination with the *in vivo* assessment of abuse potential or neurochemical effects in experimental animals, it can accurately predict new psychostimulants that may soon be clandestinely developed for black markets, providing a scientific basis for legal regulation if necessary. In addition, Ca^{2+} -assay protocols can be tailored for evaluating new types of compounds such as allosteric MAT modulators or ones that have specific therapeutic profiles at monoamine transporters.

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Abbreviations:

NPS	New psychoactive substances
PEA	phenylethylamine
PMMA	4-methoxy-N-methylamphetamine
DOM	2,5-dimethoxy-4-methylamphetamine
DA	dopamine
5HT	serotonin
NE	norepinephrine
DAT	dopamine transporter
SERT	serotonin transporter
NET	norepinephrine transporter
ADHD	attention deficit hyperactivity disorder
MDMA	3,4-methylenedioxy methamphetamine
MATs	monoamine transporters
ICSS	intracranial self-stimulation
qSAR	quantitative structure-activity relationship
PMCA	plasma membrane Ca^{2+} ATPase

NCX	Na ⁺ /Ca ²⁺ exchanger
RyR	ryanodine receptor
4-MA	4-methylamphetamine

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Fig 1.

Scheme describing Ca^{2+} fluxes in cells expressing monoamine transporters (MATs) and voltage-gated Ca^{2+} channels (Ca_V).

(A) At rest, cells keep the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{rest}}$) low and steady; the influx through leak channels and the efflux mediated by high-affinity, low-capacity extruding mechanisms (Plasma membrane Ca^{2+} ATPase, PMCA) maintain a null net Ca^{2+} flux in the plasma membrane. (B), Substrates of MATs (e.g. MDMA) induce an inward current through the transporter, depolarizing the plasma membrane, increasing the Ca^{2+} influx through voltage-gated Ca^{2+} channels, and elevating the intracellular $[\text{Ca}^{2+}]$. (C) A fraction of voltage-gated Ca^{2+} channels get inactivated reducing the initial influx, the remaining elevated influx is balanced by the efflux through low-affinity high-capacity mechanisms (Na/Ca Exchanger, NCX), reaching a new steady state ($[\text{Ca}^{2+}]_{\text{high}}$). (D) Removal of the extracellular monoamine transporter substrate reverts the depolarization, closing the voltage-gated Ca^{2+} channels, and the extrusion mechanisms reestablish $[\text{Ca}^{2+}]_{\text{rest}}$. The left panel shows the Ca^{2+} signal induced by a monoamine transporter substrate (black mark in the timeline) in cells co-expressing MATs and voltage-gated Ca^{2+} channels.

Fig 2.

DAT- mediated current, Ca^{2+} signal and DA- uptake as function of the extracellular DA concentration.

Dopamine dose-response curves are superimposed: Inward currents measured in whole-cell voltage-clamp in cells expressing DAT (thin red trace), Ca^{2+} signals measured by fluorescence microscopy in cells expressing DAT and the voltage-gated Ca channel $\text{Ca}_v1.2$ (dotted green line), and ^3H -DA uptake in cells expressing DAT (thick blue line). Data from Cameron et al. 2015 (Cameron et al. 2015).

Fig 3.

Using the electrical coupling between monoamine transporters and voltage-gated Ca^{2+} channels to characterize substrates and blockers of monoamine transporters. (Top panel) A typical experiment for assessing the effects of substrates of monoamine transporters in which Flp-In T-REx 293 cells permanently expressing DAT and transiently transfected with the voltage-gated Ca^{2+} channel $\text{Ca}_v1.2$ were loaded with Fura2 and the Ca^{2+} signals were measured using a fluorescence microscope. The measurements were performed under constant perfusion at 35°C . S(-) methcathinone (S(-)-MCAT), a substrate of DAT, produced

reproducible Ca^{2+} signals. (Middle panel) Blockers of monoamine transporters do not produce Ca^{2+} signals in cells expressing monoamine transporters and voltage-gated Ca^{2+} channels but can inhibit the signal induced by an endogenous substrate. MDPV, a strong DAT blocker, inhibits the Ca^{2+} signal induced by dopamine (DA). (Lower panel) Data collected using the Ca^{2+} -assay to compare the effects of DA and S(-)methcathinone (S(-)MCAT) as substrates and MDPV and cocaine as blockers at DAT. These are original data. For a detailed description of the methods see (Cameron et al. 2015; Solis et al. 2017).

Fig 4.

Linear correlation analysis between potencies of compounds at monoamine transporters using the synaptosome- and Ca^{2+} -assays. Red, blue, and white filled circles represent compounds tested at DAT, NET and SERT, respectively. (Left panel, linear correlation of releasers) The potency (EC_{50}) of compounds in producing signals in the Ca^{2+} -assay (x-axis) is correlated to their potency in promoting release at synaptosomes preloaded with radioactive substrate (y-axis). (Right panel, linear correlation of inhibitors) The potency (IC_{50}) of compounds in inhibiting signals induced by neurotransmitters in the Ca^{2+} -assay (x-axis) is correlated to the potency of the compounds in inhibiting uptake of neurotransmitters in synaptosomes (y-axis). The data used to perform the correlations are depicted in Table 1.

Table 1.

Potencies of several compounds for inducing Ca^{2+} signals or release (EC_{50}), or for blocking neurotransmitter-induced Ca^{2+} signals or uptake (IC_{50} ; bold values). The data here are used to calculate the correlation in Fig. 4

	Ca^{2+} -assay (nM)			Synaptosomes (nM)		
	DAT	NET	SERT	DAT	NET	SERT
N-methyl-4-MA ^a	207	252	479	41	67	67
S(+)-N-methyl-4-MA ^b	251	192	273	45	25	22
R(-)-N-methyl-4-MA ^b	3199	190	1429	476	45	175
N-ethyl-4-MA ^a	4353	728	683	795	182	102
S(+)-N-ethyl-4-MA ^b	2785	565	571	346	61	43
R(-)-N-ethyl-4-MA ^b	44490	1168	1615	4156	1190	327
N-propyl-4-MA ^a	18260	14350	2444	3064	2916	650
S(+)-N-propyl-4-MA ^b	4641	6662	872	1356	1906	197
R(-)-N-propyl-4-MA ^b	25040	26000	4280	6181	4790	3847
N-buthyl-4-MA ^a	60960	30280	Inactive	13660	6009	Inactive
Cocaine	1582	ND	ND	478^c		
S(+)-amphetamine	144 ^f	ND	ND	25 ^c		
S(-)-methcathinone	77	ND	ND	15 ^d		
MDPV	35	ND	ND	5^e		
DA	1302	ND	ND	87 ^c		
5HT	ND	ND	900 ^g			44 ^c

Data on the racemates

^a (Solis et al. 2017) and enantiomers

^b (Battisti et al. 2018) of N-alkyl-4-MA analogues were published previously;

^c previously published (Rothman and Baumann 2003);

^d previously published (Rothman et al. 2003);

^e previously published (Kolanos et al. 2015);

^f previously published (Cameron et al. 2015);

^g previously published (Ruchala et al. 2014);

ND, not determined.

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